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REVIEW ARTICLE

Creating context for the use of DNA adduct data in cancer risk assessment: I. Data organization

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Abstract

The assessment of human cancer risk from chemical exposure requires the integration of diverse types of data. Such data involve effects at the cell and tissue levels. This report focuses on the specific utility of one type of data, namely DNA adducts. Emphasis is placed on the appreciation that such DNA adduct data cannot be used in isolation in the risk assessment process but must be used in an integrated fashion with other information. As emerging technologies provide even more sensitive quantitative measurements of DNA adducts, integration that establishes links between DNA adducts and accepted outcome measures becomes critical for risk assessment. The present report proposes an organizational approach for the assessment of DNA adduct data (e.g., type of adduct, frequency, persistence, type of repair process) in concert with other relevant data, such as dosimetry, toxicity, mutagenicity, genotoxicity, and tumor incidence, to inform characterization of the mode of action. DNA adducts are considered biomarkers of exposure, whereas gene mutations and chromosomal alterations are often biomarkers of early biological effects and also can be bioindicators of the carcinogenic process.

Keywords: *Biomarkers; cancer; DNA adducts; mode of action; risk assessment*

Contents

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1. Introduction

Human health risk assessment is a process used in many regulatory decisions: pesticide registration, worker protection, development of ambient air or drinking water standards, determination of environmental remediation goals, and food safety and drug registration, to name a few. As measurement technology advances, the use of data describing steps early in the disease process has become a major trend in cancer risk assessment. These early steps are likely to be more specific to the exposures of concern and occur at lower doses than do neoplasms. Further, producing data on early steps may require fewer resources. Very importantly, data on precursor steps in cancer development may be available from human populations. Among those measurements that may be useful in cancer risk assessment processes are DNA adduct data, the subject of the present report.

At the time of publication of the U.S. National Academy of Sciences, National Research Council (NRC) framework for interpretation and verification of biomarkers (NRC, 1987, 1991; Schulte, 1989), DNA adducts were considered biomarkers of exposure, and much discussion was devoted to the requirements of validating biomarkers to serve as surrogates for more traditional measures in each of three categories: exposure, effect, or susceptibility. In contrast, mutations in the NRC context could serve as biomarkers of effect or as bioindicators[1] of the carcinogenic process. Assessment of DNA adducts can be used to characterize the metabolism and tissue dose for chemicals in different tissues, organs, and species, may provide important weight of evidence (WOE) for hazard identification and informed comparative dose-response assessment, and have been suggested to be relevant to the determination of mode of action (MOA) for carcinogenicity.

[1] A bioindicator of the carcinogenic process (bioindicator) is defined as a subset of biomarkers of effect—one that has been established to be on the causal pathway to cancer, e.g., *p53* mutation in target tissue, chromosomal translocation shown to be causative, etc.

Risk assessors are increasingly using mechanistic information and an understanding of the MOA as the basis for critical judgments on the following:

- The relevance of experimental animal data for human cancer processes;
- The condition under which a chemical may be carcinogenic in humans; and
- The presumed shapes of dose-response curves at environmental levels of exposure.

The U.S. Environmental Protection Agency's (EPA's) 2005 *Guidelines for Carcinogen Risk Assessment* (or "*Cancer Guidelines*") stressed determination of the MOA as the key step in cancer risk assessment (USEPA, 2005a). Another EPA document, the *Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens* (or "*Supplemental Guidance*"), also relies on MOA assessment in adjusting dose-response assessments to consider susceptible life stages (USEPA, 2005b).

Swenberg et al. (2008) have reviewed examples of DNA adducts as biomarkers of exposure, and of mutations as biomarkers of effect, in consideration of a MOA for carcinogenesis. They recognized the value of a 'framework analysis' for identifying needed research to better understand molecular dose and exposure-response for mutation. Our report similarly emphasizes systematic and critical evaluation of multiple types of data in order to draw inferences about the use of DNA adducts in cancer risk assessment. These types of data include, for example, mutation efficiency, dosimetry, mutation dose-response, tumor type, and tumor incidence. We also emphasize issues of database quality, reliability, relevance, and coherence.

The interpretation and integration of various measures of biological response are inherently involved in effective risk assessment for any endpoint; thus, no single biological effect or piece of data can be considered in isolation. To identify key issues for the use of DNA adduct data in cancer

risk assessment, the ILSI Health and Environmental Sciences Institute (HESI) convened a workshop in 2004 to provide a state-of-the-science overview (Sander et al., 2005). This was followed by formation of a committee whose purpose was to develop a framework for application of DNA adduct data to the risk assessment process. This DNA Adduct Project Committee held workshops in June 2005 and January 2006, and numerous meetings via conference calls, all of which led to this report.

This report discusses the current understanding of cancer as a multistep process. Next it addresses some general concepts of key events in the cancer process as well as specifics of DNA-reactive MOAs, i.e., those for which adduct data are most relevant. In Section 4, we define types of biomarkers and bioindicators that may be used as surrogates to describe internal exposure measurements and key events in that disease process. Finally, this paper presents a framework to be used in organizing and evaluating DNA adduct data in concert with data on subsequent key events. This framework stresses that adduct data should not be considered in isolation, but in the context of other toxicological data, based on the current general knowledge of the carcinogenic process as well as specific information on the chemical being assessed. We offer some general conclusions on the utility of DNA adduct data for human health risk assessment.

2. Cancer as a multifactorial process

Knowledge of mechanisms and pathogenesis of cancer has increased rapidly in the past few years with the advent of molecular biological analyses of neoplasms and preneoplastic lesions. Excellent reviews of the current status can be found in *The Genetic Basis of Human Cancer* (Vogelstein & Kinzler, 2002). Studies have shown that cancer is the end result of a multistep process through which a normal cell evolves into a malignant neoplasm. These steps may be conceptualized as occurring along the exposure-dose-response (E-D-R) continuum (see Figure 1a and Section 4 of this paper).

Another way to consider the multiple steps in the carcinogenic process is as key events in a MOA (e.g., Table 1 and Section 5 of this paper describe key events for a DNA-reactive MOA).

The definition of the various steps at the genotypic and phenotypic levels was provided initially by studies using a model for colorectal tumorigenesis (Fearon & Vogelstein, 1990). It is highly likely that tumors at other sites exhibit a similar multistep progression, although the linking of specific genetic alterations to specific phenotypic changes is not as well defined as that presented for colorectal tumors. The specific number of steps is expected to vary among tumor types. Similarly, a mutated cell is also the result of a multistep progression from the initial DNA insult to the formation of an altered DNA sequence that can be expressed as a heritable mutation.

Hanahan and Weinberg (2000) described a set of six acquired characteristics that together constitute the phenotypic changes essential for formation of a metastatic tumor from a normal cell. The characteristics are (1) unrestricted cell growth, (2) self-sufficiency in growth signals, (3) insensitivity to antigrowth signals, (4) evasion of apoptosis, limitless replicative potential, (5) sustained angiogenesis, and (6) tissue invasion and metastasis.

The emergence of these characteristics may occur in different sequences in different cancers, or be the end result of more than one insult to the cell, such as a mutation. These characteristics can be acquired through genetic (mutations at the DNA level) or epigenetic changes (transcriptional or translational changes at the DNA, RNA, or protein level). A particular cancer characteristic may also be acquired through an inherited mutation.

In summary, carcinogenesis is a complex tissue response that requires multiple steps or key events that include early events of a genetic and/or epigenetic nature, as well as later steps such as cell selection and cell-cell and cell-microenvironment interactions. Any of these steps can be affected by endogenous processes or by exposure to environmental stressors, and DNA adducts from endogenous and exogenous sources may play a role in several of these steps.

3. Mode of Action (MOA)

The EPA *Guidelines for Cancer Risk Assessment* (USEPA, 2005a) define MOA as the "sequence of key events and processes, starting with interaction of an agent with a cell, proceeding through operational and anatomical changes, and resulting in cancer formation." A "key event" is further described as "an empirically observable precursor step that is itself a necessary element of the mode of action or is a biologically-based marker for such an element." MOA differs from "mechanism of action" which constitutes a more detailed understanding and description of events, often at the molecular level (Wiltse & Dellarco, 1996). Some examples of MOAs for carcinogenic action include mutagenicity; mitogenesis; inhibition of apoptotic cell death; cytotoxicity with compensatory cellular proliferation; hormonal perturbation; and immune suppression (USEPA, 2005a). MOAs other than mutation do not, by definition, involve direct DNA interaction and modification. These non–DNA-reactive mechanisms are not addressed in this report, although this is not meant to diminish their relative importance and potential roles in tumor initiation and development. The key events framework for DNA-reactive chemicals is shown in Table 1 (modified from Preston and Williams, 2005), along with examples of biomarkers of exposure and early biological effect, and bioindicators for each key event along the cancer pathway. Some frameworks do not include dosimetry when defining key events of response at the target tissue. However, all approaches to identifying the MOA recognize the need to characterize dosimetry processes when estimating the biologically relevant dose at the target; these include absorption, metabolism (both activation and detoxification), distribution to the target tissue, and excretion. Key events are discussed in greater detail in Section 5.

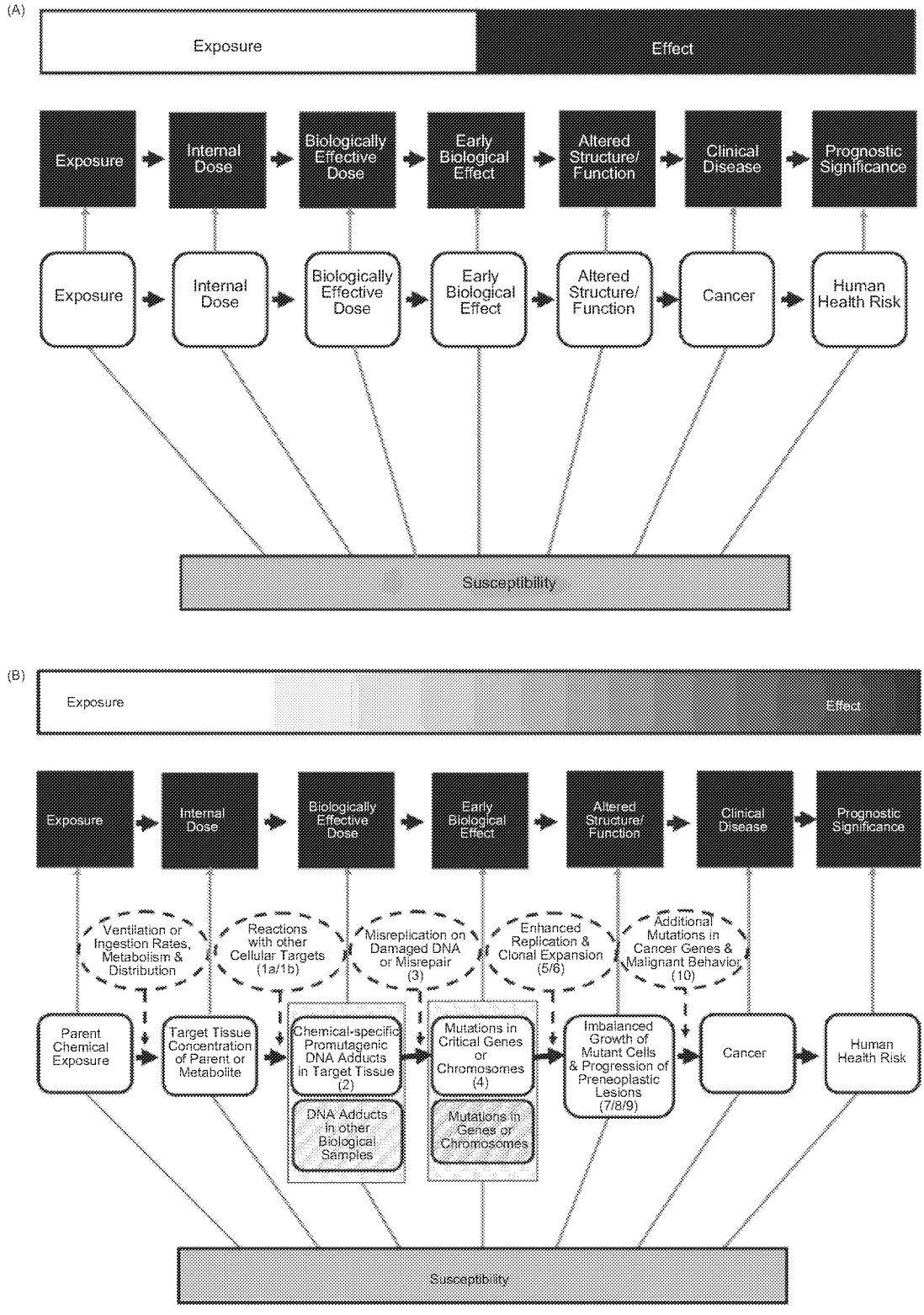

Figure 1. (A) NRC scheme for biomarker components in sequential progression (arrows) along pathogenesis between exposure and disease (black boxes) and adaptation of this scheme for application to risk assessment (white boxes) (NRC, 1987; NRC, 1991; Schulte, 1989; USEPA, 1994). Biomarkers were categorized as markers of exposure, effect, or susceptibility and could fall in more than one category. The grey lines from susceptibility indicate that factors affecting susceptibility occur along the entire exposure-dose-response continuum. (B) Revised biomarker scheme to address role of DNA adducts in DNA-reactive mode of action (MOA) for cancer. Ovals represent parameters or processes determining transitions between major components. Numbers in parentheses refer to key events defined in Table 1. The gradation rather than strict delineation between exposure and effect is deliberate to denote that this determination depends on the degree of understanding of the MOA and the nature of the available data. Components in grayed-out boxes indicate measureable endpoints that are not on the critical causal pathway as indicated by arrows, but that do inform the formation/repair of and relationship between DNA adducts and the ability to induce mutations.

Table 1. DNA-reactive MOAs: key events for carcinogenesis[a]

Key events	Biomarker or bioindicator of key event	Examples	References
1a. Exposure of target tissue (e.g., stem cells) to electrophilic species. N.B.: in some cases this requires metabolism.	Analytical measure of parent chemical, active metabolites or active radicals, or biomarkers of their presence in target tissue.	Butadiene and metabolites quantified in rodent blood; EO quantified in rodent blood	Bechtold et al., 1995; Brown et al., 1998; Himmelstein et al., 1994
1b. Reactions with other non-DNA cellular targets that have impact on adduct fate (e.g., depletion of detoxication pathways critical to clearance).	Cytoplasmic and/or nucleophilic binding of electrophilic species	PAH; AFB1; EO: GSH depletion; PO: chemical-specific GSH conjugates in target tissue	Brown et al., 1998; Lee et al., 2005
2. Reaction with DNA in target cells to produce promutagenic damage.	[Increase in] Chemical-specific promutagenic DNA adducts in target tissue	MMS or MNU: *O*6 methylguanine [*O*6-MeG], *N*-(deoxyguanosin-8-yl)-2- acetyaminofluorene	Beranek, 1990; Kleihues and Bucheler, 1977; Williams et al., 2000, 2004
3. Misreplication on damaged DNA template or misrepair of DNA damage.	Chromosomal alterations (structural and numerical changes) and gene mutations	2-AAF, EO, and DMBA: transgenes (e.g., *Lac I*); benzene: bone marrow cytogenetics, FISH for translocations, *HPRT* (in vivo); CPP: bone marrow cytogenetics	Chen et al., 2001; Meng et al., 2004; Mittelstaedt et al., 1998; Monroe et al., 1998; Recio et al., 2004; Recio et al., 2001
4. Mutations in critical genes in replicating target cell.	Mutations in oncogenes or tumor suppressor genes	2-AAF: Sequence changes in ras, p53, loss of heterozygosity; ACB-PCR, *K- ras*; environmental tobacco smoke: *p53*	Groopman and Kensler, 2005; McKinzie et al., 2006; Nesnow et al., 1998; Pfeifer et al., 2002; Puisieux et al., 1991
5. Mutations in critical genes result in enhanced DNA/cell replication.	Mitotic figures, tritiated thymidine, bromodeoxyuridine [BrdU] or proliferating cell nuclear antigen [PCNA], Ki67 immunohisto-chemistry	DEN and 2-AAF: reduced gap junctional intercellular communication in liver preneoplastic hepatocellular alter foci	Krutovskikh et al., 1991
6. New cell replication leads to clonal expansion of mutant cells.	Histological identification of growth of abnormal cells of similar morphology/type	Identical mutations in critical genes results in monoclonal amplification as identified by cell surface markers/ receptors, e.g., CC10 in lung	Kocks & Rajewsky, 1989; Yang et al., 2004
7. DNA replication leads to further mutations in critical genes.[b]	*Histological identification of growth of abnormal cells of heterogeneous phenotype*	*Genomic instability, mutations in polymerases, additional oncogenes or tumor suppressor genes, mutator gene mutations*	Beckman & Loeb, 2005; Guo et al., 2004; Loeb et al., 2003; Venkatesan et al., 2007; Vogelstein & Kinzler, 2004
8. Imbalanced and uncontrolled clonal growth of mutant cells leads to preneoplastic lesions.	*Observed groups of abnormal cells (e.g., hyperplasia, liver foci), genomic instability*	*DEN Histochemical identification of foci of γ-glutamyltransferase (GGT)-positive cells*	Pitot, 1990, 1996
9. Progression of preneoplastic cells results in emergence of overt neoplasms, solid tumors (which require neoangiogenesis) or leukemia.	*Angiogenesis markers Nuclear/ cytoplasmic ratios; Leukemia-specific chromosomal changes, tumor-specific histopathology/clinpath*	*Vascular endothelial growth factor (VEGF) expression; tumor-specific genetic alterations*	de Fraipont et al., 2000; Mitelman et al., 1997; Seon et al., 2001
10. Additional mutations in critical genes in subpopulation of cells as a result of clonal expansion and additional mutations result in malignant behavior.	*Gross observation of abnormal mass with heterogeneous morphology*	*Tumor-specific markers for malignancy; genomic instability, tumor histopathology/clinpath, metastasis*	Gupta et al., 2005; Nowell, 1976; Poste et al., 1982

[a]Modified from Preston and Williams (2005).
[b]Italics indicate key events that are not restricted to DNA-reactive MOA, but are common to others MOAs.

The following sections discuss interpretation of DNA adduct data in the context of cancer pathogenesis, and the use of these data in hazard identification, dose-response analysis, and MOA characterization.

4. Biomarkers of exposure and effect, and bioindicators of cancer

The NRC proposed a scheme in the late 1980s for categorizing biomarkers based on molecular epidemiology (NRC, 1987, 1991; Schulte, 1989). This NRC scheme acknowledged that biomarker data often come from various levels of observation or domains, ranging from genome, to tissue, to population. It was intended as an interpretive and integrative framework, and EPA adapted it in the Agency's 1994 risk assessment methods (USEPA, 1994). The exposure-dose-response (E-D-R) continuum it used to define pathogenesis is completely consistent with the determination of key events for any MOA. According to this scheme, shown in Figure 1a, biomarkers could be classified as biomarkers of exposure, of effect, of susceptibility, or any combination of categories. The assignment of a measure to a category of

biomarker depends on the degree of understanding of its relationship to pathogenesis and the amount of supporting data (USEPA, 1994).

The extent to which a marker may be viewed as pathognomonic for a disease or as a surrogate outcome measure is based on the extent that a causal relationship between the marker and the established disease outcome measure can be established (Schulte, 1989; USEPA, 1994). The relationship between DNA adducts and outcomes traditionally used in risk assessment can range from an association that is plausible based on accepted theory to an experimentally established relationship. For example, a plausible relationship could be "Mutations can cause cancer; adducts can result in mutation; so adducts are related to cancer." Alternatively, more firmly established relationships would include causal linkage of the biomarker with a key event that was established with chemical-specific data. An approach that integrates pathology studies (ultrastructural, histochemical, cellular, and molecular) with functional methods has been recommended for establishing such causal links (Lewis et al., 2002; Rubin & Rousseaux, 1991). Ascertaining whether the association is causal or merely correlative is a critical determinant of whether a particular biomarker can be used as a substitute for a clinical effect along the pathway to disease development (Fleming & DeMets, 1996). For the purposes of this report, the "clinical disease" is cancer, and the current understanding of key events of carcinogenesis for a DNA-reactive MOA is provided in Table 1.

The concept of establishing the prognostic significance of biomarkers is analogous to that of "phenotypic anchoring" advocated for the interpretation of genomic expression information in context with altered pathology (Albertini et al., 2003; Paules, 2003; Waters & Fostel, 2004). Critical to this evaluation, and to relating variables along the E-D-R continuum (Figure 1a), is agreement on the spectrum of biomarkers that are surrogates for the particular components in the continuum. Arriving at that agreement is known as biomarker "qualification" in the regulatory arena dealing with new drugs. Essentially, such qualification is a graded, "fit-for-purpose" evidentiary process that is designed to correlate a biomarker with specific biological and clinical endpoints (Wagner et al., 2007). "Qualification" of a biomarker is defined as demonstration of its domain and validation of it as a biomarker of the proposed effect or disease. This usually requires a series of independent studies (primarily toxicological) that are then supported by clinical or mechanistic studies in different biological systems, as well as by epidemiological findings (Stevens et al., 1991). An established relationship for a biomarker with the various components along the E-D-R continuum would include knowledge of the following:

1. The association between a biomarker and a preceding exposure and/or subsequent effect;
2. The shape and slope of the dose-response for the exposure biomarker or the biomarker/bioindicator-effect;
3. The apparent threshold for the effect (which may be equivalent to a no-observed adverse effect level [NOAEL]); and
4. The positive predictive value of the biomarker of exposure, biomarker of effect, or bioindicator of disease.

The predictive value of a given DNA adduct as a biomarker of exposure must be established before it can be used as a substitute for an exposure concentration in classical epidemiological research, and prior to its use for risk assessment purposes (USEPA, 1994).

When evaluating biological endpoints for use in cancer risk assessment, it is also useful to further differentiate between biomarkers of exposure, biomarkers of effect, and bioindicators of cancer (McCarthy et al., 2002; Swenberg et al., 2008). Biomarkers of exposure include DNA adducts, nonspecific DNA damage (such as strand breakage), and sister-chromatid exchanges. Examples of biomarkers of both exposure and early effect include mutations in reporter genes (i.e., genes not associated with cancer development, but being used because they are easy to evaluate); mutations in other nonessential genes; chromosome aberrations and translocations; micronuclei; and aneuploidy. In these latter examples, the biomarkers of effect demonstrate both the potential for a chemical to cause genetic alterations, and provide information on the dose-response.

In contrast, some observations can be considered bioindicators. For cancer, these could include mutations in cancer-related genes, and specific chromosome translocations associated with specific cancers, identified in target tissues for tumor formation. These observations provide information on the chemical's ability to directly affect a pathway known to be involved in carcinogenesis and, as such, identify a specific mechanistic event that is in the pathway to the disease outcome. By definition, bioindicators represent a key event for tumor formation for a particular MOA. Biomarkers of early biological effect and bioindicators can be used quantitatively to describe a dose-response curve. However, bioindicators are typically farther along the causal pathway and would be considered the more informative. Another example of a bioindicator of carcinogenesis is the induction of preneoplastic lesions (Williams, 1999).

To address these considerations of different classes of biomarkers of effect, a proposed revised biomarker schema for the E-D-R continuum is shown in Figure 1b. The gradation in shading between exposure and effect shown in this new figure is meant to denote the potential for different degrees of understanding and evidence along the E-D-R continuum. The modified schema aligns the key events identified in Table 1 (numbers shown in parentheses) along the pathway to cancer, beginning with exposure and ending with overt disease. In this context, DNA adducts or mutations in genes or chromosomes are biomarkers. By contrast, the corresponding heritable bioindicators (e.g., chemically induced mutations in a cancer gene), represent key events that continue along the causal pathway towards clonal

expansion, progression, and finally cancer. In this context, DNA adducts are not bioindicators and will be described further in this framework analysis as biomarkers of biologically effective dose.

5. Key events in carcinogenesis: DNA-reactive MOAs

The remaining discussion will concentrate on what is termed a DNA-reactive MOA as defined by Preston and Williams (2005).[2] This MOA is distinguished from MOAs based on receptor-mediated effects, hormonal changes, or mitogenesis. The proposed framework for data organization recognizes that a DNA-reactive MOA may involve direct binding (adduction) of DNA by parent chemical or its reactive metabolites (including free radicals). These adducts can be formed endogenously as the result of cellular metabolism, or can result from exogenous chemical exposure.

The following operational definitions were adopted for the purposes of these sections and the ensuing discussions.

- *Genotoxicity:* A general term for DNA damage or chromosome alteration.
- *Mutation:* A heritable change in the genetic material or chromosome structure, and/or in the functional capability of a gene product.
- *Adduct:* This includes both a chemical-specific moiety (e.g., an aflatoxin or benzo(a)pyrene adduct) covalently bound to DNA (i.e., chemical-specific adducts), as well as other additions or modifications of bases that may not be specific to a particular chemical (e.g., oxidized, deaminated, *N*7-methylated).
- *Epigenetic:* A general term for processes (e.g., DNA methylation and histone modification) that establish heritable states of gene expression without altering the DNA sequence.

Key events, as defined above (Section 3), in the carcinogenicity of DNA-reactive, mutagenic carcinogens are shown in Table 1 (modified from Preston and Williams, 2005). The table does not include events that determine dose to the target tissue, but rather describes tissue-specific key events of the response to the delivered dose necessary for tumor development. The events described in Table 1 are not necessarily sequential, e.g., some can occur simultaneously. Furthermore, some events, such as those depicted in italics (key events 7 through 10), are common to MOAs other than DNA-reactive MOAs; for example, compensatory proliferation induced by cytotoxicity. Table 1 includes a generic description of biomarkers of exposure, biomarkers of effect, and bioindicators for key events, as well as specific examples. For example, for key event 2, "Reaction with DNA in target cells to produce promutagenic damage," a biomarker for such damage would be the observation of a chemical-specific DNA adduct that is potentially mispairing (Swenberg et al., 1990, 2008).

Mutations are not the direct result of DNA adducts, but rather are the end-product of a multistep progression involving cellular processing:

- Reaction with DNA
- Formation of a stable adduct
- Recognition of the adduct by the cell's replication or repair enzymes
- Misrepair of the adduct or failure to recognize and repair the adduct prior to DNA replication
- Subsequent replication of the DNA containing the misrepaired or unrepaired
- DNA adduct to 'fix' the damage and result in a stable or heritable mutation.

Subsequent cell replication leads to clonal expansion of the mutant cells (Table 1; key events 3 through 7).

Although a specific heritable genetic change can be a necessary key event for cancer, it is not by itself sufficient. A corollary is that formation of some DNA adducts could be a key event in the carcinogenic MOA of a particular chemical, but adduct formation per se is not sufficient for either mutation or cancer induction. Furthermore, the quantitative relationship between adducts and mutations in reporter genes or cancer-related genes is usually not known. If only high-dose data are available, they cannot be strictly applied to risk assessment without further characterization at lower exposures.

6. Organizational framework for information and data needs for using DNA adduct data in cancer risk assessment

This section presents an organizational framework for the types of data typically available, and how to consider DNA adducts in that context. An association between DNA adduct formation and the observation of cancer is more likely to be considered as supporting causality if the evidence comes from several high-quality studies covering a range of doses, including the doses that cause cancer. An evaluation of the experimental DNA adduct data should consider whether or not the apparent associations have been assessed in reliable and reproducible assays (Himmelstein et al., 2009). The data evaluation in support of a MOA must be biologically plausible; this means that there is a defined MOA based on formation of the adduct(s) as the initial key event, and that this MOA is consistent with overall knowledge of the biological system. Some questions to pose are:

- Have the adducts been evaluated for their ability to induce specific mutations?

[2] DNA reactivity is defined as the formation of covalently bound adducts of the administered chemical in DNA of cells in the target tissue for carcinogenicity.

- Are the adducts chemically stable?
- How are the adducts repaired?
- How does the dose-response relationship for DNA adduct formation and metabolite formation in different organs compare with the site and dose-response for neoplasia?

6.1. *Overview of available data*

The first aspect to consider is the comprehensiveness of the database. The types of data and information that are relevant for risk assessment include, but are not limited to, the following broad categories:

- Dosimetry (physicochemical properties, and ADME[3] and pharmacokinetic data)
- Acute toxicity tests or accidental exposures
- Repeat-exposure toxicity tests in laboratory animals (subacute to chronic [2-year] bioassays)
- Genotoxicity assays across various phyla (*in vitro* to *in vivo*)
- Mechanistic studies
- Reproductive and developmental toxicity tests in laboratory animals
- Human epidemiological studies and clinical trials.

Most often, DNA adduct data are collected for chemicals that have already been evaluated in a set of tests designed to screen for the potential toxicity of the chemical for a broad array of hazards. DNA adduct data are also generated in mechanistic research studies or after exposure to a chemical has been shown to be associated with an elevated incidence of cancer in a specific target tissue of experimental animals or humans. Some data sets cover more of the E-D-R continuum (Figure 1b) than others and thus may be more convincing in establishing whether an adduct is a biomarker of exposure that may contribute to the induction of cancer.

The support for use of DNA adduct data in risk assessment will be strengthened if the chemical is also shown to be mutagenic or to affect cell growth signaling pathways associated with cell transformation. This is particularly true if these effects are observed at doses that are reasonably small multiples of potential human exposures. In general, a larger range of observations across the E-D-R continuum, and correspondence of the slopes of such responses, leads to a greater confidence in the characterization of the role of the DNA adduct in carcinogenesis. Conversely, highly discrepant slopes between DNA adducts and biomarkers of effect/bioindicators reduce the confidence of using DNA adducts in quantitative risk assessment.

[3] Absorption, distribution, metabolism, and elimination.

A variety of methods may be used to measure DNA adducts (Himmelstein et al., 2009), and the data generated may sometimes be qualitative in nature rather than quantitative. Qualitative data include demonstration of the presence of DNA adducts in the absence of structural identification or quantitative evaluation (e.g., qualitative ^{32}P- postlabeling evidence). In other cases, there may be a quantitative assessment of total DNA adducts (e.g., quantitative ^{32}P-postlabeling evidence) with no information on adduct identification or no information on the ability of the adduct to lead to a mutation or a genetically altered cell. These data provide an indication of the capability of chemicals to react covalently with DNA and serve as a dosimeter of exposure, but they may not be of further use in the estimation of risk. Ideally, the specific adducts will not only be identified, but their relative concentrations and dose-response characteristics will also be defined. Such data would be most useful for any eventual estimation of risk.

One reason to identify the specific adducts formed is that not all adducts are mutagenic. The type of adduct, and the cell type in which it is formed, can profoundly influence the mechanisms and efficiency of its repair, and the likelihood that it would lead to a mutation. Examples of adducts not considered to be promutagenic include small *N*7- alkylguanine adducts such as *N*7-methylguanine, *N*7-hydroxyethylguanine, and *N*7-hydroxypropylguanine (Boysen et al., 2009; Albertini & Sweeney, 2007). Thus, studies that identify the specific adduct(s) have greater use in risk assessment than studies that identify only total adduct levels without characterizing them.

To be useful for risk assessment, adduct levels should be related through toxicokinetic data to a therapeutic dose or to an environmental exposure; this should include consideration of steady-state conditions representing a balance between adduct formation and removal. Thus, for example, adduct data obtained from *in vitro* experiments only at concentrations not achievable in experimental animals or humans are not useful for quantitative risk assessment. Another important consideration is whether an adduct forms only as a consequence of xenobiotic chemical exposure, or also as the result of endogenous processes or from normal metabolic or dietary components (Swenberg et al., 2008).

6.2. *Dosimetry*

Dose and dose-rate are critical determinants of adduct profiles, tissue location of the adducts, and, in some cases, the type of cancer resulting from exposure. Anatomical, physiological, and biochemical factors associated with the portal of entry and the target tissue influence the chemical's uptake, subsequent distribution, and tissue response dynamics such as metabolism and detoxication. Important species/sex differences and tissue-specific aspects of these factors have been demonstrated, and include ventilation rate, cell types and their distribution, and the metabolic

rates and capacities of the different tissues. The stability of the DNA-reactive electrophile often determines if it is likely to be restricted to the tissue in which it is formed or has the potential for systemic distribution. The major categories of dosimetry data available for evaluation include the physicochemical properties of the chemical and toxicokinetic parameters.

6.2.1. Physicochemical properties of the chemical

A chemical's physicochemical properties are critical to the characterization of the potential tissue location and type of toxicity resulting from exposure. Physicochemical properties affect absorption and initial distribution of the parent compound, and thus availability for adduct formation and tissue location of the adduct. Water solubility and reactivity are two key factors that suggest the potential for portal of entry effects, especially for inhaled chemicals (Dahl, 1990; Miller & Kimbell, 1995; USEPA, 1994). The relative degree of lipophilicity versus hydrophilicity, and the molecular weight and conformation of a chemical, influence if and where a chemical is likely to be absorbed in the gastrointestinal tract.

6.2.2. Toxicokinetics

DNA adducts are formed by reactions of an electrophilic form of a chemical with a nucleophilic site in DNA (Miller & Miller, 1979). The toxicokinetic factors of absorption, distribution, metabolism, and elimination (ADME) are important determinants of whether and where adducts are formed. Metabolism of inactive parent compounds can lead to reactive intermediates, and such metabolic activation can be tissue- or cell-specific. If the parent compound is the reactive electrophile, DNA adducts can form at the site of exposure. Their formation at distant sites from their absorption or metabolism requires that the electrophile is stable enough to survive systemic distribution, or that there be distant tissue with metabolic capability to activate the parent. In this case, metabolism and elimination can play detoxication roles. Alternatively, metabolism of active parent compounds can lead to inactive intermediates, and such metabolic inactivation can be tissue or cell specific. These factors have an impact on whether an observed tumor is likely in a given tissue following exposure to a specific chemical, as well as on the shape of the dose-response curve. Such dosimetry considerations are general to any toxicity evaluation and have been discussed in detail elsewhere (Bond & Medinsky, 1995; Dahl, 1990; Miller & Kimbell, 1995; Schlesinger, 1995; Slikker et al., 2004a, 2004b; Snipes, 1995; USEPA, 1994, 2005a).

6.2.3. Target tissue versus nontarget tissue distribution

The presence of chemical-specific adducts in the target tissue for tumorigenicity is considered the most relevant finding for risk assessment. The finding of DNA adducts in tissues in which no increases in neoplasia occurred may also be informative. The presence of such adducts clearly indicates that the tissue was exposed to the active form of the chemical of interest. Thus, the adducts would be useful as biomarkers of exposure. Adducts in nontarget tissues serve as an important reminder of the multiple steps required to progress from adduct to mutation(s) to tumor, the tissue specificity of these steps, including tissue rates of cell proliferation, and the multifactorial nature of the carcinogenic process. At the present time, there is insufficient information to determine how the presence of nontarget tissue adducts should be evaluated, other than as biomarkers of exposure and dis[illegible]tion. The presence of adducts in tissues that do not form tumors also precludes the use of DNA adducts, alone, to predict tumor locations or the potency of the tumor response.

6.3. DNA adduct characterization

This section addresses the organization and evaluation of data on DNA adducts in more detail.

6.3.1. General adduct profile information

Careful consideration of the following types of information is necessary to adequately characterize and evaluate the presence of a chemical-specific DNA adduct in context with the other available toxicity information.

6.3.1.1. Data quality, reliability, and relevance. As with other data, DNA adduct information suitable for risk assessment must be generated using scientifically acceptable methods and analytical tools for adduct identification and quantitation (Himmelstein et al., 2009). Thus, in deciding whether or how to use DNA adduct data, there must first be an evaluation of data quality. Quality measures include both reliability and relevance. Reliability addresses the extent to which the procedures were documented and whether the information was obtained using methods with established reproducibility, so that the data can be used with confidence. The relevance of the data, on the other hand, relates to the extent to which the data are important for the effect of interest, in this case, tumor initiation. Both concepts of reliability and quality are readily generalized for application to DNA adduct data. For example, data collected under Good Laboratory Practices (GLP), a set of specific conditions to be met during data collection and analysis that verify data integrity, can provide support of data reliability; however, having GLP data may not address the issue of data relevance. Klimisch and coworkers (Klimisch et al., 1997) introduced an approach that addresses the quality of toxicological studies, and this approach can be applied to the evaluation of studies reporting DNA adducts. Considerations would include experimental design, sensitivity and specificity of the methodology, data evaluation criteria, and reproducibility of findings. Factors in the determination of the utility of the data include whether or not the adduct data were reproducibly derived using more than one methodology (fluorescence, mass spectrometry, radiolabeled ^{32}P-postlabeling, etc.), or reproducibly obtained in more than one laboratory. The accompanying report (Himmelstein et al., 2009) provides a detailed review of methodological considerations for determination of reliability of DNA adduct data.

6.3.1.2. Endogenous or background adducts. DNA damage can result from chemicals formed during normal cellular metabolism, and from everyday exposure to chemicals in the environment such as those present at low levels in molds, charcoal-grilled food, or automobile exhaust. DNA damage also results from everyday low-level radiation exposure. The first-listed source—normal cellular metabolism—results in what is generally called endogenous DNA damage, whereas the others are referred to as background damage. The endogenous or background adducts produced can be identical to adducts formed by exposure to exogenous chemicals. For example, *S*-adenosylmethionine, a methyl donor in the cell, produces various methylated adducts that are also formed in animals exposed to DNA-reactive carcinogens, such as dimethylnitrosamine and methyl methanesulfonate (Swenberg et al., 2008). Nitrosated bile salts are carboxymethylating agents. Ethylene produced from gut microbial metabolism and lipid peroxidation is further metabolized to ethylene oxide (EO), which produces 7-(2-hydroxyethyl)guanine (N7-HEG), which is also formed by environmental ethylene and ethylene oxide exposure (Rusyn et al., 2005; Walker et al., 2000). The major aldehydes of lipid peroxidation *in vivo* are crotonaldehyde, acrolein, 4- hydroxynonenal and malondialdehyde (MDA); each of these can react with DNA to form exocyclic adducts and 7-(2-oxoethyl)guanine, which are also formed by vinyl chloride and ethylcarbamate (Morinello et al., 2002; Nair et al., 1999).

Oxidation of the sugar moiety in DNA leads to the formation of reactive base propenals, which produce cyclic adducts (Zhou et al., 2005). Reactive oxygen species (ROS) are continuously formed during normal metabolic reactions, and they can oxidize DNA, generating several different oxidized bases and single- and double-strand breaks (Swenberg et al., 2008; Williams & Jeffrey, 2000). Many of these reactions also readily occur during DNA isolation in the laboratory, resulting in an artifactual induction of oxidative DNA damage (Himmelstein et al., 2009). ROS are one of the major sources of endogenous or background DNA damage, and 8-oxo-deoxyguanosine (8-oxo-dG) is the most studied oxidative DNA damage product. Glyoxal and methylglyoxal, which are reactive carbonyl species originating from glucose metabolism or lipid peroxidation, also produce adducts; methylglyoxal may be a component of beverages (e.g., coffee) and foods. Various age-related bulky adducts (I-compounds) of unknown origin have been reported in rodent and human tissue DNA. In addition, catechol derivatives formed metabolically from endogenous estrogens can produce rapidly depurinating bulky adducts by direct reaction, or 8-oxo-dG and MDA-derived adducts via redox-cycling (Cavalieri & Rogan, 2006).

The types and amounts of endogenous or background DNA adducts in a given tissue depend on many factors, including:

- Rates of cellular respiration or metabolism;
- Relative ratio of oxidative and antioxidative reactions; and
- Certain pathological conditions (e.g., viral infections such as colds, hemochromatosis).

The levels and kinds of endogenous or background DNA adducts can also vary substantially among different tissues and species (De Bont & van Larebeke, 2004; Gupta and Lutz, 1999; Nath et al., 1996). Total endogenous/background DNA damage in the human liver is estimated to include about 10–13 lesions per 10^6 nucleotides (nt), about half of which are contributed by abasic sites (Gupta & Lutz, 1999). Abasic sites alone represent 50,000 DNA lesions per cell, so that in total, each cell contains approximately 100,000 endogenous or background DNA lesions (Nakamura & Swenberg, 1999).

These endogenous or background adduct levels are comparable to the levels achieved by exposure of experimental animals to potent carcinogens. For example, doses that produced 50% liver tumor incidences in rats (TD50) were calculated to result in 0.5 and 20.8 adducts per 10^6 nt for aflatoxin B1 and dimethylnitrosamine, respectively (Otteneder & Lutz, 1999). The adduct level at the TD50 for 2-acetylaminofluorene (AAF) in mouse liver was 55.4 adducts per 10^6 nt.

Consideration of these data raises the question of whether or not the high levels of endogenous or background adducts may play a role in the incidence of spontaneous tumors in rodents and humans (Swenberg et al., 2008). Although damage levels reported in older publications are likely to be overestimates, especially of oxidative adducts such as 8-oxo-dG (ESCODD 2003; Himmelstein et al., 2009), more accurate data are rapidly becoming available. Unknowns that need further clarification include such issues as differential distribution of endogenous or background damage versus damage induced by a test chemical, and the effect of damage across coding and noncoding regions of the DNA (Pottenger et al., 2004).

6.3.1.3. Type of adduct formed. Most DNA-reactive carcinogens form one of five major types of electrophiles, i.e., carbonium, nitrenium, diazonium, aziridianium, and episulphonium (Williams et al., 2007), although a wide variety of bioactivation processes have been described (Guengerich, 1990). The adducts formed by a specific electrophile (or free radical) can differ depending upon its site of reaction in DNA. Depending on dose, most, if not all, DNA-reactive chemicals form several types of adducts. Oxidative DNA damage and repair intermediates, such as abasic sites and single-strand breaks, can also be formed from chemical-specific interactions and can have impact on the fate of chemical-specific adducts. It is critical to have sufficient knowledge of the type(s) of DNA damage that are formed by the chemical of interest because adducts differ in their in mutagenic potency and efficiency of repair (discussed below). For example, the risk assessor should differentiate among the following:

- Chemical-specific versus endogenous/background adducts;

- Bulky adducts that disrupt helix structure (certain adducts of 2-AAF and B[a]P) versus small adducts (MeG) that do not disrupt the helix;
- Covalent adducts versus other modifications such as oxidation of bases, abasic sites, etc.; and
- Adducts formed at base-pairing sites (e.g., O^6G) versus non–base-pairing sites (e.g.,N7G).

6.3.2. Dose-response relationship for adduct levels

Formation of chemical-specific adducts under any dosing regimen is qualitative evidence of DNA reactivity. Moreover, data at doses below those used in the specific carcinogenicity studies can provide an indication of the internal or biologically effective dose. However, because of the effect of endogenous DNA damage on the mutagenic process and the multistep nature of that process, the dose-response for induced mutations may not reflect the dose-response for chemical-specific DNA adducts (Swenberg et al., 2008). For use in dose-response assessment, it is necessary to determine the extent to which an adduct, as a biomarker, reflects recent or past exposures, peak as opposed to integrated exposures, and cumulative rather than noncumulative biological exposure or effects (Checkoway & Rice, 1992; Rundle, 2006).

The advent of ^{32}P-postlabeling and mass spectrometry methods for DNA adduct quantitation has made possible analysis of tissues after repeated dosing that more closely mimics exposures used in cancer bioassays (Swenberg et al., 2008). Exposure considerations may be different for chemicals that require metabolism to generate a DNA-reactive electrophile (or free radical); in this case, the molecular dose of DNA adducts reflects metabolism-driven kinetics and often shows saturation (La & Swenberg, 1997). Likewise, saturation of detoxication and DNA repair processes can be reflected in dose-response studies. The most informative data come from studies employing repeat dosing similar to carcinogenicity studies. For the well-studied hepatocarcinogen, diethylnitrosamine, for example, adducts in rat liver reached a steady-state level with time, and were proportional to dose (Swenberg et al., 1990).

Different DNA adducts have different half-lives, and the same adduct may have different half-lives in different tissues (Goth & Rajewsky, 1974). This leads to a requirement for varying exposure durations in order to attain steady-state concentrations. For example, recent studies have shown that two adducts that form both endogenously and following exposure to vinyl chloride have vastly different half-lives: 7-(2-oxoethyl)guanine has a $t1/2$ of approximately 4 days, whereas N^2,3-ethenoguanine has a $t1/2$ of approximately 140 days. Therefore, N^2,3-ethenoguanine will not achieve steady state until dosing is continued for approximately 40-fold longer than that required for 7-(2-oxoethyl)guanine (Mutlu et al., personal communication). With AAF, repeated treatments were necessary to produce measurable levels of adducts at very low doses, such as are typical of human exposures to carcinogens. In these dose-response studies, liver DNA adducts were formed at doses lower than those that produced other effects, such as cytotoxicity and enhanced cell proliferation (Williams et al., 2000).

In addition to chemical-specific adducts, carcinogens can elicit formation of DNA adducts indirectly by other processes, such as oxidative stress (Cadet et al., 1997; Williams & Jeffrey, 2000). Oxidative damage to DNA has been reported for a variety of carcinogens (Kasai, 1997), but usually the DNA damage results from oxidative stress generated by toxicity at the high doses used. For some carcinogens, both chemical-specific adducts and oxidative damage to DNA are observed. In such cases, the oxidative stress may serve to enhance the effect of the chemical-specific DNA adducts (Park et al., 2008; Dybdahl et al., 2003).

6.3.3. Stability, repair, and persistence

An important factor influencing mutation frequency is the process of repair of DNA damage. Such repair can occur by various mechanisms that effectively remove DNA damage, including DNA adducts. These mechanisms are base and nucleotide excision repair, recombinational repair, methyltransferase-mediated repair, and mismatch repair (Friedberg et al., 2005). In addition, DNA adducts can be bypassed during replication by a tolerance process involving lesion bypass polymerases that are error-prone. The specific type of repair process recruited and repair kinetics can depend on many factors including , (Kunz et al., 2009; Waters et al., 2009; Donigan & Sweasy, 2009):

- The nature of the adduct (e.g., *N*-(deoxyguanosin-8-yl)-2-aminofluorene versus *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene; ethyl versus methyl for N7G or O^6G);
- The particular base adducted;
- The position of the adduct on the base (e.g., O^6-alkylguanine versus *N*3- alkylguanine);
- The location of the adduct in the DNA (transcribed versus nontranscribed strand) and its adjacent nucleotides (i.e., the sequence context);
- Chromatin structure and modifications;
- Overlapping repair patches;
- The tissue or cell type in which the adduct forms; and
- The number of adducts (saturation of repair).

Different repair replication processes have different probabilities for the insertion of incorrect bases (Friedberg et al., 2005). This is a factor in the likelihood that a particular adduct will result in a mutation, and its efficiency of formation. Among the repair pathways that are known in mammalian cells, both nucleotide and base excision repair (NER; BER) can recognize and repair certain adducted bases, and have been the most extensively studied. Although these pathways are extremely efficient and operate with a high level of fidelity, errors in nucleotide insertion can occur (i.e., the incorrect base, or too few or too many bases), which can lead to

mutations, depending on further cellular processing. Repair of small alkylated lesions is typified by the O^6-methylguanine methyltransferase (MGMT) system. When DNA is alkylated at positions such as the O^6 of guanine, the alkyl group can be transferred to a cysteine residue on the repair protein. This restores the normal DNA base structure and, at the same time, causes the protein to be inactivated (suicide inactivation). As a result, repair by this error-free pathway is rate-limited by the constitutive level of the protein.

There is a family of DNA 'bypass' polymerases that are capable of translesion synthesis. This repair pathway effectively allows the DNA replication fork to bypass a lesion prior to its having been repaired in order to continue replication. These 'bypass' polymerases, by definition, act on adducts that block replication, do not remove the adduct, and are low-fidelity ('error-prone').

DNA adducts are repaired or lost at vastly different rates. Among the most rapidly repaired/lost are the *N*3-alkyladenine adducts and the rapidly depurinating estrogen catechol adducts, which reach steady-state levels in one day. *N*7-alkylguanine adducts have half-lives of 4–7 days (Boysen et al., 2009), whereas N^2,3-ethenoguanine has a half-life of approximately 140 days (Mutlu et al., in preparation). The ability to observe adducts is a function of duration of the study and the age of the animals. Adducts in the target tissue could typically have accumulated prior to observation of tumors.

DNA damage can elicit responses that arrest cell proliferation, directly facilitate DNA repair, or initiate cell death (apoptosis). An extensive signaling network has evolved in the DNA damage response pathway to prevent cells with damaged DNA from continuing in the cell cycle before repair can occur. Unrepaired adducts in cells that undergo proliferation can lead to mutations through their misreplication or, in the event of significant damage, can induce cell death (apoptosis), thereby removing the affected cells from the mutant cell pool.

6.3.4. Mutagenic efficiency

For the present discussion, mutagenic efficiency refers to the probability that a DNA adduct or an adduct load converts a normal cell to a viable cell containing a heritable alteration in the DNA (i.e., a mutation). Not all DNA adducts are equally efficient at giving rise to mutations; for example, some adducts result in altered bases that will cause miscoding upon DNA replication (e.g., O^6-methylguanine), whereas other adducts are in non–base-pairing positions in the DNA helix and are not considered to be promutagenic (e.g., *N*7-methylguanine) (Delaney & Essigmann, 2008). The efficiency of conversion of a promutagenic DNA adduct to a mutation is dependent upon a variety of biochemical and physiological processes and/or factors:

- The type and chirality of the adduct;
- The cell-, tissue-, or organ-specific DNA repair and metabolism;
- The local DNA sequence context of the adduct;
- The lability/stability of the adduct;
- The extent of disruption induced in the helix structure;
- The capacity of the affected cell to replicate to form a clone; and
- Other cellular processes such as the frequency of cell replication.

A bulky DNA adduct that has the capability to alter the DNA structure is readily detected by the DNA repair machinery and may be removed prior to replication of the DNA. An adduct present in a terminally differentiated cell, i.e., one that is incapable of replication, is not a threat for heritable mutations in that cell; however, it could represent an impediment to transcription, which could affect cell function. In addition, cellular recognition of an adduct load may drive a cell to apoptosis instead of replicating its DNA to produce a mutation. Thus, detection and quantification of DNA adducts, alone, is not sufficient to predict the induction or extent of a mutagenic response. To be most useful in risk assessment, the DNA adducts should be structurally identified and their mutagenic capabilities defined. It should be recognized, however, that there is a background rate of gene and chromosomal mutations, which could vary among cell types. These mutations are a function of the total exposure of a cell to DNA damage—including endogenous, background, chemical-specific damage, and replication errors on undamaged DNA—and its response to these events. As exposures to DNA-reactive chemicals are reduced, the biology driving mutations becomes dominated by the endogenous or background DNA damage, so that the chemical-specific adducts have a reduced impact on the induction of mutations (Swenberg et al., 2008).

Several examples in the literature indicate that certain types of DNA adducts have a higher probability of causing cell death rather than mutations (Beland & Kadlubar, 1990; Casciano, 2000). Likewise, studies with specific chemicals and their stereoisomers have shown that, although a reactive metabolite may adduct to a specific base, the biological response is dependent on the chirality of the adduct and the local DNA sequence context (Latham et al., 1993; Moriya et al., 1996; Kanuri et al., 2001).

The DNA repair pathway that recognizes the adduct and its response rates are major factors in the promutagenicity of adducts. One study indicated that aflatoxin B1, which forms DNA adducts in both neonatal and adult tissues, induced tumors only in the neonate (Fu et al., 1998). This was presumed to be a result of the replication capacity and relative rate and efficiency of repair in the neonate versus the adult, thereby allowing for a higher probability of conversion of an adduct to a mutation. Sidransky et al. (1961) reported that male rats were more sensitive to 2-acetylaminofluorene–induced liver cancer than females, and, subsequently, Katayama et al. (1984) demonstrated that this was due to greater initiation in males compared to females. Beland

Table 2. Organizational framework for information and data analysis for the use of DNA adduct data in cancer risk assessment

Dosimetry
- Physicochemical properties of the chemical
- Toxicokinetics
- Target tissues versus non-target tissue distribution

Adduct characterization
- General adduct profile
  - Data quality and reliability
  - Endogenous or background adduct levels
  - Type of adducts formed
  - Tissue responses for adduct formation
- Dose-response for adduct levels
- Stability, repair and persistence
- Mutagenic Efficiency
- Phylogenic representation

Genetic alterations (*Note:* Evaluation of these data needs to distinguish between effect in critical genes versus reporter genes— see discussion in Section 4)
- Gene mutations
- Chromosome mutations
- Aneuploidy
- Micronuclei
- Dose-response of genetic alterations
- Sequence analysis of mutations

Epigenetics and other adaptive or toxic effects
- Cell proliferation
- Cytotoxicity

Tumor data
- Laboratory animal data
  - Target tissue
  - Tumor type(s)
  - Dose-response
- Epidemiological data
  - Target tissue
  - Tumor type(s)
  - Dose-response

Evaluation of coherence and extrapolation premises
- Coherence across species, sex, and target tissue sites
- Multiple independent studies with consistent results
- Coherence across scales of observation: genome to whole tissue
- Relevance of observations to target context
  - Mammalian versus non-mammalian
  - *In vitro* to *in vivo*
  - Dose-response data

et al. (1985) reported that repeat dosing with *N*-hydroxy-2-acetylaminofluorene led to formation of three adducts, two that were acetylated and one nonacetylated, in male liver; only the nonacetylated adduct was found in female liver. The authors suggested that the acetylated adducts were critical for hepatic tumor formation, although the influence of adducts on initiation, promotion, or both was not determined. Based on the report of Katayama et al. (1984), it seems likely that the critical adducts play a role in tumor initiation. These studies provide further evidence that DNA adducts are not equivalent to mutations. Therefore, even if DNA adducts are detected in a relevant *in vivo* system, evaluation of the carcinogenic risk requires knowledge of their quantity, structure, repair characteristics, and mutagenic (and possibly cytotoxic or mitogenic) potential, and how these variables relate to levels of endogenous or background adducts and mutations in the same tissue.

6.3.5. Comparison of data from assay systems representing different phyla

It is necessary to consider species-specific characteristics of a test system when evaluating DNA adduct data for relevance to humans. It is reasonably presumed that DNA adduct data obtained from the many available assays, involving many different species, do not have equal predictive value for human outcomes. For example, the frequency of DNA adducts and the probability of their conversion to mutations is likely to be very different in microbial cells, *Drosophila*, mammalian cells *in vitro*, and mammalian cells *in vivo*. This variety in mutagenic outcome is a consequence of, for example, divergent nuclear structure (among all three phyla), differences in cell cycle duration (among all three phyla and in mammalian cells *in vitro* versus mammalian cells *in vivo*), or variable DNA repair capacity, kinetics, and fidelity (among all three phyla, and mammalian cells *in vitro* versus mammalian cells *in vivo*). Unique metabolism will also lead to different responses across these different assay systems and species. In general terms, the assays that are likely to be the most predictive of effects in humans are those in species that are phylogenically closer to humans. In addition, *in vivo* assays are generally more predictive of effects in humans than *in vitro* ones. These considerations are listed as phylogenic representation and relevance of observations to the target context (humans) in Table 2.

6.4. Genetic alterations

For the present discussion, genetic alterations in somatic cells that can be involved in the cancer process are considered to be of two types:

- gene mutations (involving one or two bases, and small deletions or insertions); and
- chromosome mutations (which can be structural or numerical changes).

It is generally accepted that more than one genetic alteration is involved in the process that converts a normal cell into a transformed cell, and eventually into a metastatic tumor. For cancer to occur, these genetic alterations need to be heritable; that is, the affected cell must survive to transmit the alteration from cell generation to generation.

Gene mutations are produced by replication errors at the sites of DNA modifications (including DNA adducts). The use of site-specific modified bases and oligonucleotides to better define frequency and specificity of mutations and repair efficiency of DNA alterations was recently reviewed (Delaney & Essigmann, 2008). Chromosomal DNA damage is induced in a similar way, but may require two or more DNA alterations that can lead to DNA interactions between coincidentally replicating regions. The resulting chromosome alterations can be deletions (also referred to as 'breaks'), which tend to be cell-lethal and therefore not heritable, or interchanges (such as stable translocations), some of which can be heritable.

Aneuploidies are changes in either whole chromosome numbers (complete aneuploidy) or in chromosomal regions (partial aneuploidy). They can be produced by chemically induced effects on the chromosome segregation machinery, or during mitosis as a consequence of segregation of chromosomes containing structural alterations. The former is generally the mechanism for whole chromosome aneuploidy and the latter for partial chromosome aneuploidy. Thus, it is expected that DNA adducts can lead to partial aneuploidies through their involvement in formation of structural chromosome alterations. In contrast, complete aneuploidies arise from effects on the proteins responsible for distribution of the chromosomes to the sister cells during mitosis and cell division, rather than from a direct interaction of the chemical with DNA.

Because not all genetic alterations are heritable, they can represent two different types of biomarkers of effect. For example, micronuclei in red blood cells are evidence of a clastogenic effect, but are not heritable. As such, they belong to a subclass of biomarkers of effect best considered an "early biological effect" in Figure 1b. Accordingly, they are not placed on the causal pathway in Figure 1b and are not considered bioindicators.

Nevertheless, micronuclei in red blood cells can inform the E-D-R for mutations, a biomarker of effect. In contrast, a heritable genetic alteration that involves either a specific gene or gene region that is mutated in specific tumor types is considered a bioindicator of carcinogenesis, and would be considered a key event, as shown in Figure 1b. This kind of bioindicator, which can fall within the category of "early biological effect" or "altered structure/function," would be the best quantitative data for risk assessment, as discussed above in Section 4.

6.5. *Epigenetic and other adaptive or toxic effects*

Epigenetics is broadly defined as processes that establish heritable states of gene expression without altering the DNA base sequence. These processes include cytosine methylation and histone acetylation, each of which alter the regulation of gene expression but do not affect the base sequence of DNA (Feinberg, 2001). As described above, the accumulation of heritable changes in the genome is central to the initiation and promotion of cancer. Although mutation has long been regarded as essential for the development of tumorigenesis, epigenetic modifications have more recently been proposed as an additional critical component (Sansam & Roberts, 2006).

The relationship between DNA adduct formation and any altered epigenetic pattern is largely unknown. However, some research indicates that adducts can influence epigenetic patterns, specifically DNA methylation (i.e., formation of 5-methylcytosine). For example, DNA adduct formation due to oxidative stress or chemicals such as dimethylsulfate and methylnitrosourea can result in altered methylation. Under conditions of oxidative stress, the common DNA adduct 8-oxo-dG has been shown to interfere with the ability of the human DNA methyltransferase to methylate nearby target cytosines (Turk et al., 1995). Additionally, Tan and Li (1990) demonstrated that O^6- methylguanine located 5' to cytosine can affect the maintenance methylation of the opposite strand in a hemimethylated duplex. The presence of this adduct might destabilize the hemimethylated site and cause the methylase to detach; another possibility is that this adduct could either enhance or diminish the affinity of the site as a substrate for the methylase, depending on its position within the genome. Thus, DNA adducts can either increase or decrease the methylation of neighboring cytosines without changing the DNA base sequence, but leave an abnormal, yet heritable, epigenetic pattern.

DNA-reactive electrophiles can also react with cellular nucleophiles other than DNA, notably proteins and RNA. These reactions, as well as DNA reactions, can lead to cytotoxicity, impairment of cellular function, and loss of viability. Sufficient cell death can elicit a tissue regenerative response. Such induced cell proliferation can enhance the sensitivity of the tissue to carcinogenicity by increasing the chemical's effect per unit dose (Williams et al., 2000), because replicating cells are more susceptible to mutation induction than nonreplicating cells.

6.6. *Tumor data*

The finding of an increase in neoplasia as defined by any of several criteria establishes the carcinogenicity of a chemical (Williams et al., 2007). The patterns of the tissues affected, and the types of induced neoplasia, can provide some insight into possible MOAs (Williams et al., 2007). For example, tumors exclusively in endocrine tissues, particularly benign tumors, do not suggest a DNA-reactive MOA, although certainly endocrine tumors can be induced by DNA damaging agents. In the present context, a DNA-reactive MOA would be suggested by findings of systemic, malignant neoplasms in several tissues, in both sexes and more than one species, and with a short latency (USEPA, 2005a). For certain tumor types, DNA-reactive carcinogens typically induce preneoplastic lesions prior to the development of neoplasms (Williams, 1999). Identification of such lesions at early time points can provide information relevant to the significance of the adducts formed by these chemicals. This section discusses the considerations for these data in laboratory animals and humans.

6.6.1. Laboratory animal data

It has been suggested that the following observations indicate a DNA-reactive MOA for cancer: tumors in multiple species; tumors at multiple sites (systemic carcinogen); multiple tumor types; and reduced latency. It should be noted that when tumors are observed in a laboratory animal species, the relevance to humans of the key events and the MOA must be judged (Cohen et al., 2003, 2004; Meek et al., 2003; Preston & Williams, 2005; Seed et al., 2005). When relying on laboratory animal data alone to determine relevance of observed tumors, it is necessary to take into account species differences in dosimetry and tissue dynamics (Cohen et al., 2004; Slikker et al., 2004a, 2004b; USEPA, 2005a).

In order for an effect to be considered a key event for cancer induction, temporal concordance is important; thus adducts must be present in the target tissue prior to tumor development. Adducts present in older animals or in neoplastic tissues may not be relevant to adduct levels involved in neoplastic progression, and therefore may not be predictive of a DNA-reactive MOA. Dedifferentiated neoplasms often lose expression of bioactivation enzymes (e.g., cytochromes P450 or CYPs) and acquire enhanced chemical purging systems (e.g., multiple drug resistant [MDR] activity), which render them refractory to adduct formation. Also, neoplastic tissues may have different levels of DNA replication and repair fidelity than unaffected tissues.

In contrast to the identification of adducts, identification of predicted mutations in cancer-related genes in neoplastic tissues lends substantial support to a DNA-reactive MOA. Examination of neoplasms for mutations in cancer genes has been reported in animal studies. Although not all data on tumor suppressor gene mutations in angiosarcomas of the liver from vinyl chloride–exposed animals have demonstrated mutations in the *p53* or *H-ras* genes, examples of such mutations have been reported, making this a plausible key event. When a different mutational spectrum is found for chemically induced versus spontaneous neoplasms, such data can be useful in determining a mutagenic MOA (Barbin et al., 1997; Marion & Boivin-Angele, 1999).

It is possible that a chemical can induce neoplasms in one tissue by a DNA-reactive MOA and in another by a non–DNA-reactive MOA. For example, in rats treated with the antiestrogen tamoxifen, liver tumors were associated with DNA adducts, whereas ovarian granulosa cell neoplasms were probably due to hormonal effects (Hard et al., 1993).

6.6.2. Epidemiological data

As with induced animal neoplasms, systemically induced tumors in humans, often at multiple sites, suggest a DNA-reactive MOA. With DNA-reactive carcinogens, the latency period for tumor development can be short or long. For example, treosulfan was associated with secondary leukemias with latencies of <10 years (IARC, 1987). However, vinyl chloride–induced angiosarcomas of the liver were seen within 12–28 years of onset of high level occupational exposure (pre-1974). A longer latency of 27–47 years was evident for those who had only some high exposure pre-1974, but mostly had lower exposure from the post-1974 period, during which time exposures dropped 500-fold (ATSDR, 2006; Lewis et al., 2003).

There are numerous reports of measurement of DNA adducts in patients with cancers believed to be attributable to a specific chemical, or in groups exposed to cancer-causing substances (Groopman et al., 1993; Talaska et al., 1996; Mollerup et al., 1999; Zhao et al., 2000; Tang et al., 2001; Faraglia et al., 2003). To support a DNA-reactive MOA for any associated neoplasia, adducts that are known to be promutagenic must be identified in the target tissue of tumorigenicity. Ideal support for a DNA-reactive MOA is the observation of adduct formation prior to development of a tumor, and a dose-response relationship between adducts in target tissue and exposure.

The determination of whether or not DNA adducts are present in a suspected target tissue in human studies can be difficult to assess. Published studies on tamoxifen and endometrial cancer provide an interesting example. Endometrial adduct levels in women treated for breast cancer, some of whom subsequently developed endometrial cancer, were reported either to be very low (Hemminki et al., 1996; Shibutani et al., 2000) or undetectable (Beland et al., 2004). The discrepancy in these findings indicates a need for further evaluation in order to determine if a DNA-reactive MOA is more plausible than an alternate, non–DNA-reactive MOA, such as a hormonal effect.

6.7. Evaluation of coherence and extrapolation premises

A critical aspect of synthesizing the context for the use of DNA adduct data is consideration of the coherence across the observations and the dose-responses for the different endpoints. Coherence should consider the consistency of the findings across different species, sexes, target tissues, and adduct types. The premises required to extrapolate the dose-responses for different observations (e.g., across exposure routes, organizational scale, and species) should be evaluated for their strengths or limitations, and relevance to the human exposure scenario as the ultimate target of the assessment process. For example, the extrapolation premise required for the use of an *in vitro* assay to describe the dose-response for humans versus ex vivo or *in vivo* data in a mammalian species is not likely to carry the same WOE. Coherence of findings across the organizational scale from genome to tissue to population levels would be considered compelling evidence for including DNA adducts in the causal pathway. Multiple independent studies with consistent results also bolster the WOE.

7. Conclusions and next steps

As emerging technologies for the determination and characterization of DNA adducts become more and more sensitive (Himmelstein et al., 2009), with a concomitant increased collection of DNA adduct data, it is advisable to develop a framework for the evaluation and application of DNA adduct data to cancer risk assessment. Such a framework will allow and encourage consideration of all the available data, while enhancing the consistency and transparency of risk assessment choices. Use of a framework will also help standardize the evaluation and use of these data and serve to identify critical research and information needs.

The key events in carcinogenesis for chemicals acting via a DNA-reactive MOA are described in Table 1. In the foregoing review of the considerations supporting a DNA- reactive MOA, the following conclusions emerge:

- In animals and humans, DNA is not pristine. Adducts are formed from endogenous metabolic processes and from daily environmental insults not always attributable

to any particular chemical. The use of DNA adduct data in a risk assessment for a particular chemical exposure must include a consideration of these endogenous or background levels.

- Not all DNA adducts are promutagenic or lead to a heritable effect. The relevance of adducts for cancer risk assessment is a function of the consequences of DNA adduct formation and subsequent processing, rather than of the adducts themselves. Proper adduct analysis involves both quantitation and identification of chemical-specific adducts.
- Identification of a chemical-specific DNA adduct in the absence of tumors or other data in experimental animals or humans is suggestive of potential cancer risk and merits further investigation. However, such adduct data, by itself, can not be used for prediction of cancer or in the quantitative estimation of cancer risk.
- It is essential to relate adduct levels to a therapeutic dose, dietary intake, or to an environmental exposure, through toxicokinetic data. This includes consideration of the temporal or steady-state conditions of the disposition of the chemical or its metabolite, and the resultant level of DNA adducts that represents a balance of formation and removal.
- Data obtained from *in vitro* experiments may provide ancillary information that contributes to an understanding of MOA, but when obtained at concentrations not achievable in experimental animals or humans, these data are not useful for quantitative risk assessment.

In summary, DNA adduct data, by themselves, are informative but not sufficient for assigning an MOA for tumor development. To determine MOA, a systematic analysis of the adduct data should be made in the context of other information about the chemical and its effects on biological systems. This analysis includes types of adducts formed, tissue distribution of adducts, dose-response for adduct formation, mutagenic efficiency of the adducts, persistence and repair, and their relationship to endogenous or background adduct levels and types. Of major importance will be quantitative evidence for the induction of gene and/or chromosomal mutations. Such data will carry the greatest weight if they are on a critical gene related to the cancer, that is, bioindicators. Biomarkers of effect, e.g., mutations in reporter genes, may also be useful in extrapolating the dose-response. Other information would include dosimetry data (such as consideration of the physicochemical properties and toxicokinetics of the chemical), other adaptive or toxic effects, as well as the animal carcinogenicity data and, where available, epidemiological data in exposed humans. Coherence of the findings across species, sexes, and sites of cancer is important. Likewise, coherence of effects across the scale of observational levels (e.g., genome to tissue), and across a range of time frames, is critical to understanding both the pathogenesis of cancer and the potentially causal role of DNA adducts.

The possibility of thresholds for adduct formation or consequent mutations or tumors is an important consideration, but is beyond the scope of this particular report. Threshold considerations and calculations would, of necessity, be dependent on the type of adduct (i.e., exogenous only, endogenous and exogenous), and its efficiency of repair and mutagenesis, and these are dependent upon the chemical under study. It would be most effective to address evaluation of thresholds for adducts or their effects in the context of specific case studies. The HESI DNA Adduct Project Committee has selected chemicals for case studies. These include chemicals that produce adducts not found endogenously, and those that produce adducts that are also formed as a by-product of normal metabolism. Case-study evaluations will be especially relevant to recent discussions regarding low-dose extrapolation (White et al., 2008).

The objective of the next phase of this HESI DNA Adduct Project Committee will be to apply the organizational framework in this report to the examination of three case-study chemicals to develop a more conceptual framework and recommendations for the use of DNA adduct data in cancer risk assessment. In order to organize the array of data typically available for cancer risk assessment and for determination of a biologically plausible DNA-reactive MOA (see Table 1), the data will be evaluated according to the outline presented in Table 2. The inclusion of data-rich case studies is important for developing the framework because those case studies impart the most information from which to learn, and can be used to develop principles that can be applied to less data-rich situations. Later evaluation of less data-rich case studies will allow application of the principles and more detailed development of the framework initiated based on the data-rich examples. The framework developed from the case studies should be useful not only for organizing data, but also for ensuring that issues described in this report are considered in data evaluation.

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Abbreviations

8-oxo-dG	8-oxo-deoxyguanosine
2-AAF	2-acetylaminofluorene
ACB-PCR	allele-specific competitive blocker polymerase chain reaction
ADME	absorption, distribution, metabolism and elimination
AFB1	aflatoxin B1
ALT	alanine aminotransferase; serum glutamate pyruvate transaminase
AST	aspartate aminotransferase; serum glutamic-oxaloacetic transaminase
B[a]P	benzo[a]pyrene
BER	base excision repair
BrdU	5-bromodeoxyuridine
CPP	cyclophosphamide
CYP	cytochrome P450
DEN	diethylnitrosoamine
DMBA	7,12-dimethylbenz[a]anthracene
E-D-R	exposure-dose-response continuum
EO	ethylene oxide
EPA	United States Environmental Protection Agency
FISH	fluorescence *in situ* hybridization
GGT	γ-glutamyltransferase
GLP	Good Laboratory Practices
GSH	reduced glutathione
HESI	ILSI Health and Environmental Sciences Institute
HPRT	hypoxanthine-guanine phosphoribosyltransferase
ILSI	International Life Sciences Institute
MDA	malondialdehyde
MDR	multiple drug resistant
MeG	methyl guanine
MGMT	*O*^{6}-methylguanine methyltransferase
MMS	methyl methanesulfonate
MNU	*N*-nitroso-*N*-methylurea
MOA	mode of action
N7G	*N*7-guanine
N7-HEG	*N*7-hydroxyethylguanine
N7-MeG	*N*7-methylguanine
NER	nucleotide excision repair
NO(A)EL	no observed (adverse) effect level
NRC	National Research Council
nt	nucleotides
O^{6}G	*O*^{6}-guanine
O^6-MeG	*O*^{6}-methylguanine
O^6-HEG	*O*^{6}-hydroxyethylguanine
PAH	polycyclic aromatic hydrocarbon
PCNA	proliferating cell nuclear antigen
PO	propylene oxide
ROS	reactive oxygen species
$t_{1/2}$	half-life
TD_{50}	chronic dose in mg/kg body wt/day predicted to induce tumors in half the test animals at the end of a standard lifespan for the species
VEGF	vascular endothelial growth factor
WOE	weight of evidence

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